

# Immune Function Assays As Indicators of Chromate Exposure

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The potential immunomodulatory effects of chromium were investigated using a series of *in vitro* and *in vivo* studies. Chromium (as  $K_2CrO_4$ ) in concentrations spanning five orders of magnitude was added *in vitro* to T-lymphocyte (concanavalin A) and B-lymphocyte (liposaccharide) mitogen cultures and was found to inhibit T-lymphocyte responses at all concentrations tested and to inhibit B-lymphocyte responses at all but the lowest concentration tested (0.01 mg/L). When the same concentrations of chromium were employed in mixed lymphocyte cultures, antigen-induced thymidine uptake was inhibited at the highest concentrations (100 mg/L–1 mg/L), enhanced at 0.1 mg/L, and equal to control values at lower concentrations. Splenocytes isolated from rats exposed to  $K_2CrO_4$  in drinking water exhibited enhanced responses to T- and B-lymphocyte mitogens. The addition of 0.1 mg/L of chromium to a mixed lymphocyte culture containing splenocytes taken from chromium-exposed rats increased by 5-fold the uptake of thymidine by these cells. These increased responses of cells from chromium-exposed rats may indicate chromium-induced sensitization and may possibly be used as a biological marker for chromium exposure.

## Introduction

Although some heavy metals, notably mercury, lead, nickel, and zinc, have been shown to alter immune-associated processes (1–3), the immunological consequences of chromium exposure have not been studied extensively. We wished to determine whether or not chromium could alter certain immune function assays as part of a larger program designed to assess the utility of these assays as markers for chemical exposure. As a first approach, the effects of chromium (as  $K_2CrO_4$ ) at concentrations spanning five orders of magnitude (100 mg/L–0.01 mg/L) were assessed *in vitro* on the mitogen-induced proliferation of T- and B-lymphocytes and on the antigen-induced proliferation of T-lymphocytes in one-way mixed lymphocyte cultures. Subsequently, splenocytes from rats exposed to  $K_2CrO_4$  in drinking water were isolated, and the responses of these cells to B- and T-lymphocyte mitogens and to the proliferative stimuli in mixed lymphocyte cultures were assessed. In all studies proliferation was measured by tritiated thymidine uptake.

In the *in vitro* studies, an unremarkable dose response was observed in the mitogen assays. However, in the mixed lymphocyte cultures, thymidine uptake was enhanced at 0.1 mg/L chromium. Higher concentrations resulted in almost no uptake of thymidine, while lower concentrations had no effect on thymidine uptake. Thus, in a narrow concentration range, *in vitro* chromium apparently enhanced the proliferation of T-lymphocytes in the mixed lymphocyte culture.

In the *in vivo* studies there was more evidence for chromium-induced enhancement of lymphocyte proliferation. Splenocytes taken from chromate-exposed rats showed enhanced thymidine uptake at day 2 in both the B- and T-lymphocyte mitogen assays and in the mixed lymphocyte culture assay. Of particular note, the addition of 0.1 mg/L chromium to the mixed lymphocyte culture increased by 5-fold the thymidine uptake of splenocytes taken from chromium-exposed rats. This latter result may be indicative of chromium-induced sensitization which, if true, may form the basis of a method for assessing chromium exposure.

## Methods

Splenocytes from Fischer 344 rats (Charles River, Wilmington, MA) were used throughout the studies. Splenocytes from Sprague-Dawley rats (Charles River, Wilmington, MA) served as stimulator cells in the mixed lymphocyte cultures. Spleens were removed under sterile conditions and placed in RPMI (Gibco, Grand

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Island, NY) media. Spleens were then pressed through stainless-steel screens and the cells gently aspirated. Cells were then transferred to test tubes, and tissue clumps were allowed to settle. The supernates were washed one time and the cells in the supernates were counted.

## Mitogen Assays

Mitogen assays were performed in RPMI 1640 media. For the concanavalin A (ConA) assays, RPMI 1640 was supplemented with 5% fetal calf serum, 0.5% gentamicin, and 1% glutamine. ConA powder was added at 10  $\mu\text{g}/\text{mL}$ . For the liposaccharide (LPS) assays, RPMI 1640 was supplemented with 3 mM glutamine, 25 mM Hepes buffer, 1% 2-mercaptoethanol, 1% penicilline/streptomycin/neomycin and 5% fetal calf serum. LPS was added to this media at a concentration of 100  $\mu\text{g}/\text{mL}$ .

For both mitogen assays, cells were plated in microtiter plates at  $2 \times 10^5$  cells in 0.2 mL per well. Chromium as  $\text{K}_2\text{CrO}_4$  (J. T. Baker, Philipsburg, NJ) was added at appropriate concentrations. Cells were cultured in 8 to 12 replicates in 5%  $\text{CO}_2$  atmosphere for up to 72 hr. At 18 hr before harvesting, cells were pulsed with tritiated methylthymidine (specific activity, 20 Ci/mmol) (New England Nuclear, Boston, MA) at 1  $\mu\text{Ci}/\text{well}$ . Cells were harvested using an automated cell harvester (Cambridge Technology, Cambridge, MA) onto glass fiber filters. Each filter was placed into a mini-scintillation vial, and 7 mL of scintillation fluid was added. After dark adaptation, each vial was counted for 5 min in a scintillation counter (Wallac Oy Co., Turku, Finland).

## One-Way Mixed Lymphocyte Cultures

One-way mixed lymphocyte cultures were performed in RPMI 1640 supplemented as described for a ConA assay. Stimulator splenocytes from outbred Sprague-Dawley rats were incubated in mitomycin C (Sigma, St. Louis, MO) for 40 min at  $37^\circ\text{C}$ . These cells were then washed three times and mixed with Fischer 344 splenocytes at a ratio of 2:1 ( $4 \times 10^5:2 \times 10^5$ ). Cells were then cultured in 8 to 12 replicates in the wells of microtiter plates in 0.2 mL of media. Cells were incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere for up to 5 days. Pulsing, harvesting, and scintillation counting were performed as described above for the mitogen assays.

Animals undergoing the *in vivo* exposures to chromium were housed singly in wire mesh cages. Chromium, as  $\text{K}_2\text{CrO}_4$ , was dissolved in distilled water and placed in standard rat water bottles. Animals were allowed food (Purina Lab Chow) and fluid *ad libitum*. Control rats received distilled water *ad libitum*. Chromate solutions and water were changed weekly.

## Results

The responses of rat splenocytes to T- (ConA) and B- (LPS) lymphocyte mitogens in the presence of various

concentrations of chromium are given in Figure 1. Almost no response to either mitogen was observed until concentrations of chromium were reduced to 0.1 mg/L. At this concentration and at 0.01 mg/L chromium, the responses to the T-lymphocyte mitogen appeared to be inhibited by the chromium to a greater extent than the responses to the B-lymphocyte mitogen.

The responses of rat splenocytes to the antigenic stimuli of one-way mixed lymphocyte cultures in the presence of various concentrations of chromium are given in Figure 2. Similar to the responses observed with the mitogen-induced stimulations, there were essentially no responses observed until concentrations of chromium were reduced to 0.1 mg/L. At this concentration, there was an increase in uptake of thymidine. At lower concentrations, uptake to thymidine was equal to control values.

The T-lymphocyte mitogen (ConA) responses of splenocytes isolated from rats exposed to chromium in drinking water are shown in Figure 3. Splenocytes taken from rats exposed to 200 mg/L chromium showed depressed levels of response throughout the 3 days of culture. However, splenocytes taken from rats exposed to 100 mg/L chromium showed elevated levels of re-

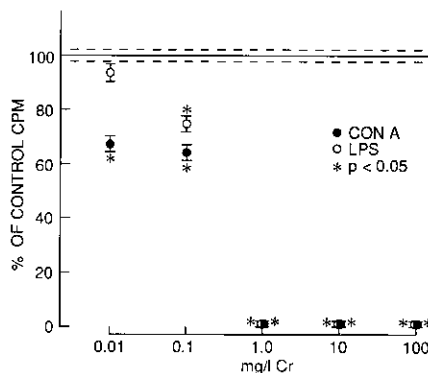


FIGURE 1. Effect of *in vitro* chromate on mitogen-stimulated proliferation.

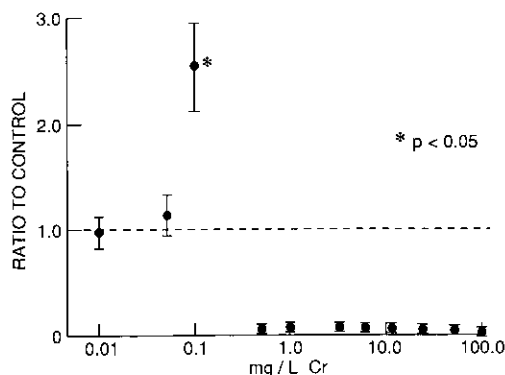


FIGURE 2. Effect of *in vitro* chromate on mixed lymphocyte response.

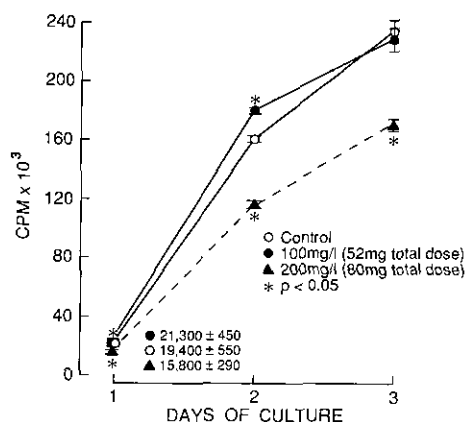


FIGURE 3. Effect of 3-week *in vivo* chromate exposure on ConA-induced proliferative activity.

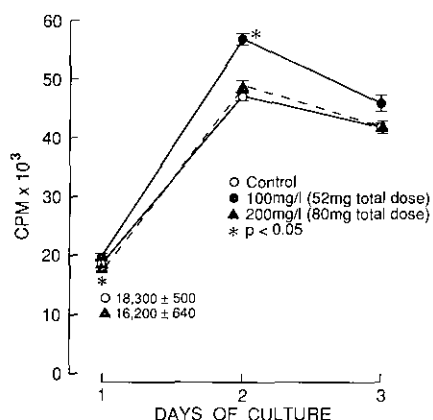


FIGURE 4. Effect of 3-week *in vivo* chromate exposure on LPS-induced proliferative activity.

sponse at days 1 and 2 of culture and response equal to that of control on day 3.

The B-lymphocyte mitogen (LPS) responses of splenocytes isolated from chromate-exposed rats are shown in Figure 4. Splenocytes taken from rats exposed to 200 mg/L chromium showed essentially the same responses as those splenocytes isolated from control rats. Splenocytes taken from rats exposed to 100 mg/L chromium exhibited an elevated response at day 2 of culture but showed responses equal to those of controls at days 1 and 3. As observed in the *in vitro* chromium experiments, lymphocytes responding to LPS appear to be more resistant to the effects of chromium than lymphocytes responding to ConA.

The mixed lymphocyte culture responses of splenocytes taken from rats exposed to 100 mg/L chromium over 10 weeks are shown in Figure 5. In this experiment the samples of splenocytes from chromium or control rats were divided, and some cells were cul-

tured in the presence of 0.1 mg/L chromium in addition to the mitomycin-c-treated Sprague-Dawley splenocytes (stimulator cells). Control cells not cultured with added chromium show a gradual diminution of thymidine uptake over the course of the culture period. However, control cells cultured with added chromium show an uptake of thymidine that is substantially higher than that exhibited by control cells cultured without added chromium. Moreover, there is no indication of a diminution of thymidine uptake over the course of the culture period in control cells cultured with added chromium. Cells taken from chromium-exposed rats and cultured without added chromium show increased uptake of thymidine relative to control cells cultured without added chromium, but there appears to be a gradual decline in thymidine uptake at days 4 and 5 of culture. In sharp contrast to the other cells and culture conditions, cells taken from chromium-exposed rats and cultured with added chromium show a marked elevation in thymidine uptake that increases dramatically during the culture period. Eventually, the uptake of thymidine by these cells exceeds by more than 5-fold the thymidine taken up by cells taken from chromium-exposed rats but cultured without added chromium.

## Discussion

A unifying explanation for these results is that chromium reacts with lymphocyte cell surface proteins, thereby altering the responses of the cells to various stimuli. In the *in vitro* mitogen assay (Fig. 1), chromium may react with the cell surface receptors for the mitogens, thereby blocking the proliferation effects of the mitogens. At the higher concentrations, chromium may also become internalized within the cell and react with proteins responsible for replication.

Chromium alteration of cell surface proteins would also explain the enhanced proliferation observed in the mixed lymphocyte reaction at an *in vitro* chromium concentration of 0.1 mg/L. At this concentration the

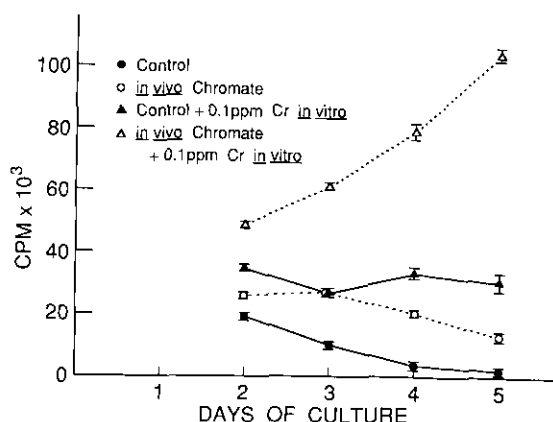


FIGURE 5. *In vivo* chromate exposure ± 0.1 ppm chromate *in vitro*.

cell surface proteins of the stimulator cells may be altered by reaction with chromium such that these cells become more antigenic, thereby eliciting a greater proliferative response by the effector cells. Alternately, proteins on the surfaces of some of the effector cells may be altered by reaction with chromium such that these cells are now antigenic and elicit a proliferation response from nonaffected effector cells.

Such alterations of cell surface protein and their attendant antigenic responses would also explain the enhanced mitogen-induced proliferation observed in cells taken from rats exposed to chromium *in vivo*. After 3 weeks of chromium exposure, populations of certain T-lymphocyte subsets could be sufficiently enlarged such that a greater response to a T-lymphocyte mitogen might be elicited (Fig. 3). The higher concentration of chromium (200 mg/L) is clearly toxic and may be lethal to the lymphocytes or block their replication by mechanisms discussed above. The lower concentration (100 mg/L), however, may induce a growth of certain T-lymphocyte populations as a result of antigenic stimuli. Because activated T-lymphocytes are involved in the activation of B-lymphocytes (4), a population of T-lymphocytes responding to chromium-induced antigenic stimuli might also expand the B-lymphocyte population. This in turn could lead to enhanced responsiveness to the B-lymphocyte mitogen LPS (Fig. 4).

Perhaps the strongest evidence for chromium-induced activation of T-lymphocytes is given in Figure 5. The responses of splenocytes taken from rats exposed to chromium and cultured with additional chromium appear to be classic sensitization responses. Ten weeks of exposure to 100 mg/L chromium may have induced a subpopulation of T-lymphocytes that expands rapidly

in the presence of chromium or a chromium hapten. Such a phenomenon has been observed in some individuals exhibiting nickel contact dermatitis (5). In these cases, clones of lymphocytes have been isolated that will proliferate in the presence of nickel ions but not in the presence of other metal ions. Thus, some individuals exhibit sensitization to nickel salts. This same phenomenon may have occurred in the rats exposed to chromium. If this is true, it may be possible to use this phenomenon as a marker for toxic metal exposure. Lymphocytes from individuals exhibiting a toxic response may be cloned and the clones grown in the presence of a number of different metal ions. Thus, it may be possible to identify a metal inducing toxic responses from blood samples taken from affected individuals. Such possibilities are under investigation in this laboratory.

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## REFERENCES

1. Berger, N., and Skinner, A. Characterization of lymphocyte transformation by zinc ions. *J. Cell. Biol.* 61: 45-55 (1974).
2. Pelletier, L., Pasquier, R., Hirsch, F., Sapin, C., and Druet, P. *In vivo* self-reactivity of mononuclear cells to T cells and macrophages exposed to HgCl<sub>2</sub>. *Eur. J. Immunol.* 15: 460-465 (1985).
3. Warner, G., and Lawrence, D. Stimulation of murine lymphocyte responses by cations. *Cell Immunol.* 101: 425-439 (1986).
4. Roitt, I., Brostoff, J., and Male, D. *Immunology*. C. V. Mosby Co., St. Louis, MO, 1985, p. 87.
5. Smigaglia, F., Scheidegger, D., Garotta, G., Scheper, R., Pletscher, M., and Lanzaveschia, A. Isolation and characterization of Ni-specific T cell clones from patients with Ni-contact dermatitis. *J. Immunol.* 135: 3929-3932 (1985).